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Apoptosis induced by *Zizyphus jujuba* seed essential oil in colorectal cancer cells line HT-29

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ABSTRACT

Background & Aim: In traditional medicine, *Zizyphus jujuba* (jujube) has been used due to its medicinal properties and various physiological functions such as antioxidant, anticancer and anti-inflammatory properties. This study was conducted to study anti-cancer and apoptotic effects of essential oil from *Zizyphus jujuba* seeds and to evaluate the effect of essential oils on p53, APC and KRAS genes expression in HT-29 colorectal cell line.

Experimental: In this study, the essential oil of jujube seeds collected from orchards in Isfahan was prepared by Clevenger apparatus and then analyzed by GC-MS. The effects of toxicity and cell viability were determined using Trypan Blue, MTT and clonogenic methods. DNA fragmentation and apoptosis techniques were used to evaluate the mechanism of the effects of essential oil on cell death by flow cytometry. Finally, Real-time PCR was used to evaluate the expression of p53, APC and KRAS genes and their role in induction of apoptosis.

Results: Essential oil of *Zizyphus jujuba* seeds reduced the viability of cells by concentration and time-dependent manner as compared to the control group. The essential oil also induced apoptosis by increasing gene expression p53 and suppressing gene expression KRAS.

Recommended applications/industries: The present results indicated that essential oil of *Zizyphus jujuba* seeds induces apoptosis by targeting genes involved in colon carcinogenesis. However, further investigations on signaling pathways are needed to fully confirm the results of this study.

1. Introduction

Colorectal cancer (CRC) is the third most frequent cancer in men and the second most common cancer in women with a survival rate of less than 5 years (Bray *et al*., 2018). It is a heterogeneous disease that occurs due to multiple and successive genetic and molecular disorders such as mutations in tumor suppressor genes such as APC, p53 and KRAS oncogene gene, which eventually leads to impaired differentiation and maintenance of genomic DNA, imbalance between growth rate and cell apoptosis, the creation of multiple pathological lesions in the intestinal mucosa and eventually tumorigenesis (Li *et al*., 2015; Abraha, and Ketema, 2016).

Despite multiple approaches for treatment such as the use of advanced surgery and chemotherapy, current therapies available are not sufficiently effective and their efficacy is limited by the side effects of the drugs and drug resistance in colon cancer. This highlights the need to focus on safer and more effective approaches to discovering new compounds among natural resources

with chemotherapeutic properties. Numerous studies have reported that biologically active compounds isolated from plants have anti-proliferative and anti-cancer effects against colon cancer cells (Aiello *et al*., 2019).

The common *Ziziphus jujube* (*Z. jujuba*) is a high-nutrient food belonging to the Rhamnaceae family that can thrive in various climates and can grow naturally in some regions of Asia, having two main species., including Ziziphus mauritiana. Lam and Ziziphus jujube Mill (Taechakulwanijya *et al*., 2013). Previous studies have shown that the jujube fruit extract is a good source of various bioactive compounds that alone or in combination with other herbal formulations have shown numerous anti-cancer effects in several human cancer cell lines (Tahergorabi *et al*.,2015). Numerous studies have reported that *Z. jujuba* seeds have several medicinal properties, including sedative and anti-anxiety hypnotic activities (Li *et al*.,2019) and essential oil from *Z. jujuba* (EOSZ) are reported to have anti-inflammatory and antibacterial activities (Al-Reza *et al*.,2010; Al-Reza *et al*.,2010).

The purpose of this research was to evaluate the antiproliferative and apoptotic properties of *Zizyphus jujuba* seed essential oil (EOSZ) and to analyze the expression of APC, p53 and KRAS genes in HT-29 cell lines in vitro.

2. Materials and Methods

2.1. Plant material.

Ripe fruits of *Z. jujuba* were gathered from Isfahan province, Iran, in September 2019. It was then transferred to the herbarium of Isfahan Science University and their morphological features were matched with the existing herbarium sample. The seed was separated from the fruit flesh, cleaned away from the dry sunlight, ground and kept in optimum conditions.

2.2. Isolation of essential oil

The seeds were air dried and powdered (100 g) and then hydro-distilled using a Clevenger-type apparatus for 4 h. The essential oil obtained was transferred to a dark glass container and then stored at 4ºC for further analysis. The essential oil yield (percentage) was calculated based on weight obtained from the dry weight of plant material.

2.3. Gas Chromatography/Mass Spectrometry (GC-MS)

The essential oil of *Zizyphus jujuba* seeds was analyzed by Gas Chromatography/ Mass Spectrometry (GC-MS). Qualitative analysis was completed using a Hewlett-Packard 6890 gas chromatography device equipped with a mass detector (Hewlett-Packard model 5973 HP). The column used was HP-5MS with a diameter of 25 mm, film thickness of 0.25 µm and a length of 30 m. The column temperature was initially programmed at 40 °C, and increased to 200 °C at 8 °C/min and finally to 290 °C at 40 °C/min and held at this temperature for 3 min. The essential oil sample (2 µl) was injected in a ratio of 1: 90 into the device. A power of 70 electron volts was used for ionization and mass spectrum. The retention indices (RI) of the compounds were calculated using a series of homologous n-alkanes injected under equal conditions of the samples. In order to identify the essential oil components, the main peaks of the sample mass spectrum of the essential oil were compared with the standard spectra provided by the electronic spectroscopy library on the instrument software (Adams, 2007). The amount of essential oil constituents was obtained and reported as retention time (Rt) and peak area (Area%).

2.4. Cell culture

HT-29 human colon adenocarcinoma cells code NCBI C154 was acquired from the National Cell Bank of Iran (Pasteur Institute, Tehran-Iran). Cells were first counted and percentage of cell survival determined in DMEM culture medium containing 10% FBS (bovine fetal serum) and antibiotics (100 U/ml Penicillin, 100 µg/ml Streptomycine, Gibco) deemed as complete medium in 25 ml flasks. Flasks were incubated at 37 °C, 5% CO_2 and 95% humidity. Cell culture medium was changed every 48 h and were divided once a week.

2.5. Cell viability assay using MTT Test

After the cells reached 80% confluency, they were trypsinized, neutralized and diluted with culture medium, and were seeded in each well (1×10^4 cells/well) containing 100µl complete DMEM medium with 10% FBS in a 96-well plate) and allowed to attach to the bottom of the plate overnight at 37°C in a 5% CO_2 humidified incubator. When the cells adhered to the bottom of the plate by reverse phase microscopy,

the supernatant and any suspension was removed and the cells were treated with fresh medium containing different concentrations of essential oil from *Zizyphus jujuba* seeds (100, 200, 400, 600, 800 and 900 µg /ml). After 24, 48 and 72, about 15 µl of MTT solution was added to the wells and after 4 h of incubation about 100 µl of dimethyl sulfoxide (DMSO) was added in each well for dissolving the formazan pellets and then read at the wavelength of 570 nm. Percentage of cell proliferation and survival in each sample compared to control wells of 100% living cells was calculated and determined.

Percent cell proliferation = (A treated)/ (A control) × 100.

Concentration of essential oil that inhibits cell growth up to 50% (IC50) is expressed as cytotoxicity. All tests and analysis were performed in three copies and average values were recorded.

2.6. Colony formation assay

Colony formation is an *in vitro* test for cell survival and proliferation based on the cell's ability to form Colonies. Single-cell suspensions of exponentially growing cultures were seeded in 6-well plates at a low density (1,000 cells per well) and allowed to adhere to the bottom. The cells were treated with different concentrations (0, 400, 600 and 800 µg/ml) of essential oil (EOSZ) for 24 h. After that, the treated cell medium containing the essential oil was replaced with fresh medium. The cells were then incubated at 37 °C for 15 days and then the cells were washed with phosphate-buffered saline, fixed with methanol and stained with 0.5% crystal violet. Colonies with a diameter of more than 50 cells were counted using a microscope with 10 to 40 times magnifications depending on colonies morphology and size (Crowley *et al.*, 2016). The experiment was repeated three-times.

2.7. Analysis of DNA fragmentation using Agarose gel electrophoresis

Apoptosis is characterized by fragmentation of genomic DNA into 180 to 200 bp oligonucleosomal fragments. The DNA ladder method was used to visualize and view apoptotic endonuclease degradation products. This test is a qualitative method and has a tracking level of about 5000,000 cells and is used in cell cultures as well as tissues. DNA was extracted by culturing about 1200 human colon adenocarcinoma cells in each of the 6-well plate wells with DMEM supplemented with 10% FBS and antibiotics. After 6 hours of incubation, the cells were treated with different concentrations (0, 400, 600 and 900 µg/ml) of EOSZ. After 24 hours, the wells were washed twice with PBS and collected in tubes with 500 µl of lysing buffer and 10 µl of protein kinase K followed by overnight incubation at 50 °C. Then, about 40 µl of saturated NaCl (5 M) was added and after mixing well, were placed at 4 °C for 10 min. Centrifugation was carried out for 12 minutes at 12,000 rpm and the supernatant was transferred to the microtubule and 1 ml of 100% cold ethanol was added to each of the microtubules. The samples were centrifuged again for 15 min at 12000 rpm and after complete removal of ethanol, about 1 ml of 70% ethanol was added and the samples were centrifuged again by pipetting and mixing well for 10 min. After complete removal of ethanol, the samples were allowed to dry at room temperature for 20 min. Finally, about 5 µl of 6X DNA loading buffer was added and DNA samples were run in 1.5% agarose gel dry wells in TAE containing 0.5 µg/ml ethidium bromide and visualized with UV illumination using a Gene Flash gel documentation system (Green, Sambrook, 2019).

2.8. Flow cytometry assay

This technique which is widely used to identify and evaluate cell death was used to determine apoptosis. In this method, PI fluorescent dyes are used to emit fluorescent light as well as light scattering. The extruded phosphatidylserine (PS) residue can be identified on the outer membrane of apoptotic cells using Annexin V and Propidium Iodine (PI) to detection of cells with late apoptosis and necrotic with characteristic plasma membrane and nucleus destruction. Briefly, about 1×10^{6} of the HT-29 cells were cultured into each petri dish (30 mm) in DMEM medium containing different concentrations of EOSZ (0, 400, 600 and 900 µg/ml) and incubated at 37º C. After 48 h, the cells were trypsinized and washed twice with cold PBS, first resuspended with 100 ml of binding buffer, then 5 ml of FITC Annexin V and 5 ml of PI). After 15 min RT in the dark, 400 ml of binder buffer was added. The samples were then analyzed using FACS calibur (BD Bioscience, USA) using Cell Quest Pro analysis software (Becton Dickinson, USA) (Crowley *et al.*, 2016).

2.9. Real-time PCR

For determination of p53, KRAS and APC gene expression, 5×10^5 cells per well were seeded into 6-well plates of HT-29 cells containing DMEM medium and after reaching 80% confluency for 48 hours were treated with or without 400, 600, and 800 µg/ml of EOSZ. Cells were collected and total RNA was obtained from the treated and control cells according to the protocol using Gene All kit. The integrity was determined by 1% gel electrophoresis and the purity and concentration of RNA were detected using a Nano-Drop spectrophotometer (ND-1000TM, Thermo Scientific) at 260 nm wavelength. 1 µg of RNA samples with a light absorption ratio of 260:280 nm between 1.8-2, according to the instructions of the 2-step PCR-RT kit for cDNA synthesis (Aldrich-sigma, UK). The primers were designed using Gene Runner software, and the correct binding of the primers to the relevant sequence was investigated, and the binding of the primers to other sequences was investigated by the Blast program at NCBI prepared by Sina Clone Company. The sequence of primers presented in Table 1. After examining the melting curve and evaluating the efficiency of the primers and plotting the standard curve, the gene expression was evaluated based on the cycle threshold (Ct) and relative expression levels were determined as $2^{-[\Delta\Delta C(t)]}$. Each sample was analyzed in triplicate, and data were stated as mean fold changes ± standard deviation (SD) for three different amplifications.

Table 1. List of primers, nucleotide sequence and melting point of primers.

Primer Name	Forward 5` to 3`	Reverse 5` to 3`	Tm	
			Forward	Reverse
P53	GCCCAACAACACCAGCTCCT	CCTGGGCATCCTTGAGTTCC	60.88	60.76
KRAS	CTATTCGCAGCTCACACAGTTTAC	TTCTTAATTTGGTCTGCGGC	60.11	60.18
APC	GACTGGTATTACGCTCAACTTCA	CAATTGCCTTCTGGTCATATCTG	60.05	60.08
GAPDH	AGGGCTGCTTTTAACTCTGG	CCCCACTTGATTTTGGAGGG	59.04	60.27

2.10. Statistical analysis

For analysis of all data in this investigation, the average ± SEM of 3 individual samples was presented. Student t-test was used to compare groups in experiments, and p-value <0.05 was considered statistically significant. All data analysis was performed using SPSS 20 software.

3. Results and discussion

The light essential oil of *Zizyphus jujuba* seeds was obtained with the yield of 0.05 % (v/w) based on the dried weight of ground seeds. Total of 24 different compounds representing 92.4% of the total oil were identified. The chemical composition of jujube seeds essential oil and the percentage of composition, molecular formula, molecular weight and retention indices of the constituents are given in Table 2 while the compounds are listed in order of their elution on column DB-5. The major constituents of the oil were eugenol (49.37%), isoeugenol (9.60%) and eucalyptol (5.38%). Caryophyllene oxide (2.93%) and benzaldehyde (2.34%) have been previously reported as the main compounds of the essential oil of *Zizyphus jujuba* seeds (EOSZ) which is almost consistent with the findings of this study (Al-Reza *et al*., 2009).

Table 2. *Phytocomponents* identified in essential oil of *Zizyphus jujuba* seeds by gas chromatography-mass spectrometry.

NO.	Name of the compound	RI	Peak area (%)
1	Hexanoicacid	973	0.32
2	Benzaldehyde	997	2.93
3	Eucalyptol	1039	5.38
4	Linalool	1063	0.24
5	Benzyl acetate	1097	0.69
6	Octanoic acid	1193	0.41
7	Chavicol	1243	0.62
8	Cinnamaldehyde	1263	2.63
9	Cis-β-ocimene	1284	1.04
10	Eugenol	1411	49.37
11	Isoeugenol	1436	9.60
12	α-Humulene	1455	3.84
13	Ledol	1467	0.73
14	5-Cadinene	1473	0.61
15	Tetradecanoic acid	1489	0.08
16	Dodecanoic acid	1491	0.36
17	Eugenol acetate	1495	2.53
18	β-Caryophyllene	1511	6.83
19	Cuparene	1523	0.36
20	Minoxidil	961	0.13
21	Veridiflorol	1628	0.31
22	Caryophyllene oxide	1623	2.16
23	trans-α-bisabolene epoxide	1741	0.90
24	Lin oleic acid	2184	0.26

CRC cells have common characteristics with other cancer cells, such as uncontrolled growth, resistance to apoptosis, angiogenesis, and insensitivity to growth inhibitors (Koosha *et al.*, 2019). Evaluation of sensitivity and viability of HT-29 cells by using EOSZ at different concentrations in three-time intervals of 24, 48, and 72 hours was shown in Table 3. It was found that decreased cell viability can be directly related to EOSZ concentration and time-dependent IC_{50} values (concentration leading to 50% survival) are presented in Table 3 for all the 3 time periods.

Therefore, concentrations of 600 µg/ml (IC_{50} of 48 h), 400 µg/ml (low dose) and 800 µg/ml (high dose) were used for subsequent experiments. Then, in order to evaluate results of EOSZ treatment on the clonogenicity of HT29 cells, the assay was performed by using standard criteria for a colony (greater than 50 cells or greater than 60 microns in diameter), increasing the concentration of essential oil inhibited the formation of colonies and reduced the size of HT-29 colonies. It was observed that the concentration of 400 µg/ml reduced colony formation by about 20% and had little effect on colony size, while at 600 µg/ml, it reduced colony formation and colony size by about 60%. At a concentration of 800 µg/ml, essential oil inhibited the formation of colonies and reduced the size of the colonies by up to 94%. The results showed that the colonization rate of HT29 cells after EOSZ treatment was regularly densified compared to the control group (Fig. 1). However, in this study, the results of these methods have confirmed each other. In a study on THP-1 monocyte / macrophage cells, essential oil obtained from *Zizyphus jujuba* seeds inhibited cell growth and prevented cell migration (Dehnavi *et al.*, 2018). In another study, ethanolic extract of jujube seed showed anti-growth and cytotoxic effects on HL-60, HeLa, Jurkat and Molt-4 cells without cytotoxic effect on normal cells (Mishra *et al.*, 2011; Taechakulwanijya *et al.*, 2016). Previous studies on *Z. jujuba* fruit extract demonstrated a significant antiproliferative effects on human hepatoma cells (HepG2) (Huang *et al.*, 2007), human larynx carcinoma (HEp-2), human cervical carcinoma (HeLa) and T cell leukemia (Jurkat) (Vahedi *et al.*, 2007) and human thyroid carcinoma cell lines (C643) cancer cells (Dabaghian *et al.*, 2018).

Table 3. Inhibitory effects of HT-29 cell line treated with *essential* oil of *Zizyphus jujuba* seeds for 24, 48 and 72 h.

Essential oil concentration (µg/ml)	Inhibition (%) ± SD		
	24 h	48h	72h
100	8.23 ± 2.53 *	6.78 ± 1.43	10.84 ± 2.45 **
200	14.33 ± 2.11	18.10 ± 2.06*	32.93 ± 3.15
400	27.18 ± 1.53*	30.44 ± 1.59	52.44 ± 2.13*
600	36.50 ± 2.12	53.24 ± 2.12*	64.29 ± 1.45
800	48.22 ± 2.17**	58.48 ± 3.15	73.49 ± 2.28*
900	59.18 ± 1.12*	67.23 ± 2.59	80.42 ± 2.10
IC_{50}	836± 3.21	621 ± 1.45	406 ± 2.31

Each value is expressed as mean ± standard deviation (n = 3). * represent $p < 0.05$, ** represent $p < 0.01$ compared with untreated cells.

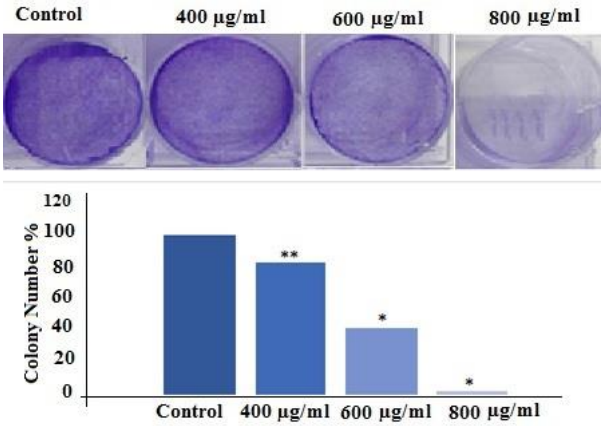

Figure 1. *In vitro*, colony formation efficiency of *Zizyphus jujuba* seed essential oil in HT-29 cancer cells (clonogenic method; 14 days). Cell clonogenesity was significantly inhibited by EOSZ treatment in HT-29 cell line. The data are expressed as mean ± SD of colony formation efficiency compared with the control (three replications). * represent $p < 0.01$, ** represent $p < 0.001$ compared with untreated cells.

DNA is an important molecular target for the destruction of tumor cells and an important biochemical feature of the apoptotic process (Majtnerová and Roušar, 2018). HT-29 cells were treated with EOSZ (0, 400, 600, 800 µg/ml) for 48 h, and it was found that essential oil treatment led to the destruction of internucleosomal DNA into small pieces which confirmed apoptosis. The explicit diffusion bands of DNA fragments represent several times the internal DNA length of the nucleus cells (about 180-200 bp). The apparent density of DNA fragment bands increases with increasing concentration of EOSZ in treated groups but no apoptotic DNA ladder was

observed in control cells for 48 h (Fig. 2). This effect of EOSZ can be explained by the ability of this essential oil to stimulate and induce apoptosis and thus prevent DNA unwinding and replicating. In human Jurkat leukemia T cells, ethanolic extract of jujube seed caused significant internucleosomal DNA generation in a time- and concentration-dependent manner (Taechakulwanijya *et al*., 2013).

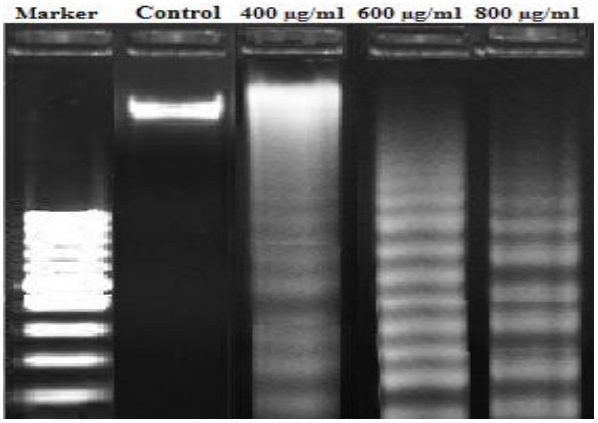

Figure 2. DNA fragmentation of EOSZ-treated apoptotic HT-29 cells (0, 400, 600, 800 µg/ml) for 48 h.

Apoptosis is a programmed and non-random physiological method for cell death that can be distinguished from necrotic death by Annexin V-FITC/ propidium iodide (PI) staining. Apoptotic cells are degraded by binding to annexin to foreign phosphatidylserine, and necrotic cells are characterized by PI passing through the membrane and binding to DNA (Dai *et al*., 2009). In annexin V/PI and flow cytometric studies, it was found that inhibition of cell growth and proliferation in the treatment with EOSZ on HT-29 cancer cells was induced by apoptosis. As shown in Figure 3 (A- a and A-b), in control HT-29 cells, approximately 97.14% of the cells were alive, 0.91% were early apoptotic and 1.54% were cells in the secondary necrotic stage or late apoptosis, while according to Figure 3A (b), 3A (c) and 3A (d) the percentage of live cells after treatment for 48 h with concentrations of 400 *µ*g/ml, 600 *µ*g/ml, and 800 *µ*g/ml of the EOSZ, were 62.31%, 48.21%, and 24.41%, respectively. The percentage of cells with primary apoptosis were 4/74%, 9.38%, and 14.61%, respectively, and the percentage of cells with late apoptosis were 28.32%, 37.02%, and 46.35%. Finally, the percentage of necrosis cells were 4.63%, 5.39%, and 9.34%, respectively. Treatment of colorectal cancer cells with essential oil of *Zizyphus jujuba* seed showed that apoptosis was observed at all concentrations used. This ratio of apoptosis was concentration-dependent so that as the concentration of essential oil increased, the number of apoptotic cells increased. At concentration of 600µg/ml (IC_{50}, 48 h), apoptosis was observed to be about 50% higher than control group. These results confirm the validity of MTT assays, clonogenic assay and Trypan blue staining and DNA fragmentation. These results showed that EOSZ induced cell apoptosis which was dose-dependent.

Numerous studies on different cancer cell lines *in vitro* or on animals *in vivo* have shown that essential oils and their compounds possess various activities such as inhibition of growth and proliferation and activation of the cell death pathway by apoptosis or necrosis. This can be due to its antioxidant and lipophilic nature, as well as the low molecular weight of essential oils, which allow them to cross cell membranes and cause numerous changes in the cell (Andrade *et al*., 2018; Tuttolomondo *et al*., 2013). In fact, it can be assumed that EOSZ is lipophilic in nature which enables it to enter the cell by crossing the cell membrane.

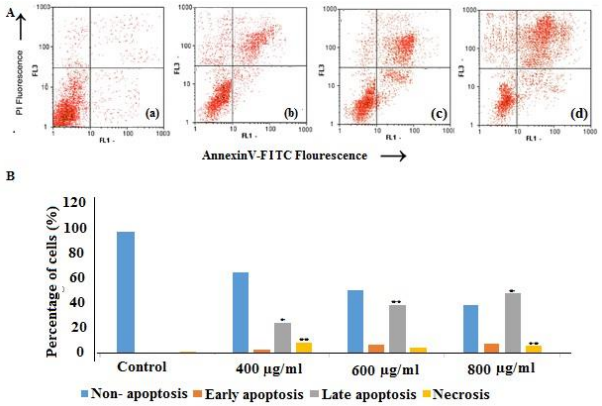

Figure 3. Induced apoptosis analyzed by flow cytometric analysis of HT-29 cells by treatment with concentrations of 0, 400, 600, 800 µg/ml *Zizyphus jujuba* seeds essential oil of after 48 h using Annexin V-FITC / PI method. Figure A: histogram view of induction of apoptosis (a) control group; (b) treatment with 400 µg/ml EOSZ; (c) treatment with 600 µg/ml EOSZ and (d) treatment with 800 µg/ml EOSZ in HT-29 cells and histogram classification as healthy cells (Annexin V−, PI−), primary apoptotic cells (Annexin V +, PI−), late apoptotic cells (Annexin V +, PI +) and damaged cells (Annexin V−, PI +). Figure B: the percentage of viable, apoptotic and necrotic cells between different experimental groups. Data represent the mean ± SEM of at least three independent experiments (** $P< 0.05$, * $P< 0.01$).

On the other hand, studies have shown that mutations in the tumor suppressor genes such as APC, p53, and KRAS oncogenes will lead to dysfunction of the apoptosis mechanism. Subsequently, with the ineffectiveness of the apoptotic system and its failure to destroy mutated colon cells with high proliferation rates, it causes the accumulation of mutated colonic cells, and the formation of the initial adenoma, which may lead to adenocarcinoma and eventually CRC (Testa *et al*., 2018). Evaluation of APC, KRAS and p53 gene expression after EOSZ treatment in Figure 4 showed that essential oil treatment increased the expression of p53 tumor inhibitor gene and also decreased KRAS oncogene gene expression in a concentration-dependent manner while treatment with EOSZ had no significant effect on APC gene expression. Activation and expression of the p53 gene in colorectal cancer can activate apoptotic pathways through various signaling pathways (Li *et al*., 2015). On the other hand, mutations in the KRAS gene in colorectal cancer have been shown to disrupt signaling pathways associated with cellular apoptosis and its general regulation (Kawakami *et al*., 2016).

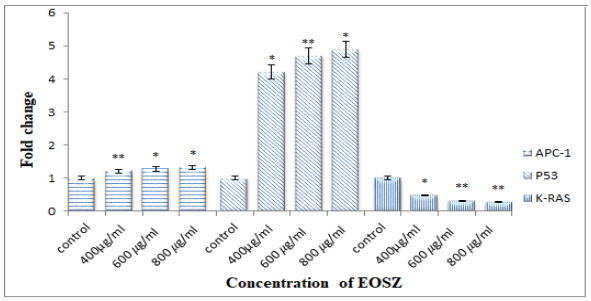

Figure 4. Expression of APC, KRAS and p53 genes in HT-29 colorectal cancer cell line treated with essential oil of *Zizyphus jujuba* seeds for 48 h at concentrations of 0, 400, 600 and 900 µg/ml using real time PCR. The data are expressed as mean ± SD of three independent experiments. * represent $p < 0.05$, **represent $p < 0.01$ compared with untreated cells.

According to gas chromatographic findings, the main constituents of EOSZ include eugenol with 49.37% and isoeugenol with 9.60%. Several studies have shown that eugenol has anti-proliferative properties and stimulates and induces apoptosis in several categories of cancer cells, including mast cells, melanoma, skin tumors, osteosarcoma, leukemia and gastric cancer cells by regulating the expression of the gene p53. Its molecular mechanisms associated with the expression of the gene p53, such as inhibiting the NF-κB pathway or reducing the expression of the H-ras and Bcl2 genes (Jaganathan and Supriyanto, 2012). In the HT-29 and HCT-15 cell categories, treatment with eugenol destroyed the mitochondrial membrane potential, ROS production and activation, increased expression of the p53 gene and eventually induction of apoptosis (Jaganathan *et al*., 2011).

Therefore, it can be assumed that most of the cytotoxicity and apoptotic properties of the EOSZ are related to the two phenolic compounds of the essential oil, eugenol and its isomer, isoeugenol.

4. Conclusion

The findings showed that essential oil of *Zizyphus jujuba* seeds exhibits antitumor and apoptotic properties through the expression of genes involved in the carcinogenicity of colorectal cancer p53 and KRAS, indicating that these effects may be related to essential oil compounds, especially eugenol. However, further studies to separate the compounds and to investigate the signal link paths are needed to confirm the present results.

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